REMARKS

Claims 1, 4 and 11 have been amended herein. Claims 6, 7, and 12-21 have been canceled. Such cancellation is without prejudice on the merits to further prosecution of these claims in one or more continuing applications. The claims as amended find support in the claims as originally filed. Claims 1 and 11 have been amended to recite that the streptokinase encoded by the construct had an amino acid sequence as encoded by SEQ. ID. NO: 3. Claim 4 has been amended to include the subject matter of Claim 7 (now canceled). No new matter has been added.

Claims 1--5 and 8-11 remain in the case.

Favorable reconsideration is respectfully requested.

Rejection of Claims 1, 2, 4-5, and 7-11 Under 35 USC §112, First Paragraph:

This rejection is believed to have been overcome by amending Claims 1 and 11 to recite that the construct encodes a streptokinase wherein the amino acid of the streptokinase is as encoded in the DNA of SEQ. ID. NO. 3. By adding the structural link to "SEQ. ID. NO. 3" in independent Claims 1 and 11, Applicants submit that the rejection of Claims 1, 2, 4-5, and 8-11 Under 35 USC §112, first paragraph has been overcome. Withdrawal of the same is respectfully requested.

Rejection of Claims 1-5, 8-9, and 11 Under §102(b) in View of Pupo et al. (1999) *Biotechnology Letters* 21:1119-1123:

As applied to Claims 4, 5, and 8-10, this rejection is believed to have been overcome by appropriate amendment to Claim 4. Specifically, Claim 7 (now canceled) was not made subject to this rejection. Claim 4 has been amended to include the subject matter of Claim 7. Therefore, Applicants respectfully submit that with respect to Claims 4, 5, and 8-10, this rejection is no longer tenable. Withdrawal of the same is respectfully requested.

As applied to Claims 1, 2, and 11, this rejection is respectfully traversed. The Examiner's characterization of the Pupo et al. reference is not logically sound. The

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Examiner states (at the top of page 6 of the Final Office Action) that Pupo et al. teach that "most of the insoluble [*sic*] SKC fraction could be washed off with buffers, but the reference fails to say that all of said insoluble [*sic*] fraction was washed off in its entirety." (Emphasis in the original.) As a factual matter, Pupo et al. never said the SKC activity present in the "insoluble fraction" was itself insoluble. Pupo et al. wrote that "the low amount of SKC in the insoluble fraction" was caused by contamination with "soluble SKC." See Pupo et al., 1122, left-hand column, first paragraph. There is no ambiguity in this statement. Pupo et al.'s "insoluble fraction" did not contain "insoluble" SKC as alleged by the Office. Pupo et al's "insoluble fraction" was contaminated with "soluble SKC." This soluble contamination could be largely removed by washing with PBS.

The Examiner's conclusion that the SKC activity remaining in the insoluble fraction is therefore "insoluble" is not supported by the clear language of the Pupo et al. reference. Pupo et al. does not say that the SKC activity found in the "insoluble fraction" is insoluble. Pupo et al. cleary state that the SKC activity is a contamination from the soluble fraction. Pupo et al. go on to state that most of it was removed by washing with PBS. The remaining SKC activity could have remained there for any number of reasons totally unrelated to solubility, including adsorption to disrupted cell fragments. In other words, had Pupo et al. been a little more careful in separating the soluble fraction from the insoluble fraction, the contamination would not have happened in the first place, and Pupo et al's insoluble fraction would have been devoid of SKC activity.

Note also that the present claims require that the construct drive the expression of enzymatically active inclusion bodies. The Pupo et al. reference is totally silent with regard to the expression of enzymatically active inclusion bodies. On this point, it cannot be assumed that a protein expressed as an inclusion body is active because most proteins when expressed as inclusion bodies are inactive and denatured. See Exhibit A, attached hereto and incorporated herein. Exhibit A is product literature

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from BioNobile Oy, Turku, Finland for a kit for purifying His-tagged proteins from inclusion bodies. See page 1, left-hand column.

Lastly, the Office states that "Pupo's intention in expressing its streptokinase is, in fact, irrelevant" (Office Action, page 5). Applicants respectfully disagree. Pupo et al. teach a method of expressing **soluble** SKC and state that an "insoluble fraction" was found to contain a small amount of SKC activity. But the authors go on to state that the SKC activity in the insoluble fraction was due to contamination of this fraction with **soluble** SKC (p. 1122, left hand column, first paragraph). Pupo et al. go on in that same paragraph to state that "most of this protein could be removed by washing several time with PBS." Thus, nowhere do Pupo et al. teach that the insoluble SKC is anything more than an inconvenient byproduct of their disclosed method. Thus, Pupo et al. cannot be considered an anticipatory reference because a valid anticipatory reference must **teach**. Pupo et al. do not teach anything regarding making insoluble and enzymatically active streptokinase. They teach only that one should be careful in separating the soluble fractions from the insoluble fractions so as not to cause cross-contamination. For instance, in *In re Wilder*, 429 F.2d 447, 166 USPQ 545 (C.C.P.A. 1970) the CCPA stated that a prior art reference:

> may yet be held **not** to legally anticipate the claimed subject matter if it is found not to be sufficiently enabling, in other words, if it does not place the subject matter of the claims within the possession of the public.

Here, Pupo et al. do not enable the public to fabricate insoluble SKC in the form of an enzymatically active inclusion body. Pupo et al. merely show in Fig. 2, lane 6 that the insoluble cell fragment may be contaminated by **soluble** SKC.

Further, nothing in Pupo et al. teaches whether the SKC found in the "insoluble fraction" was enzymatically active. Applicants claim an expression construct producing **enzymatically-active** streptokinase in the form of an inclusion body. There is no discussion of the activity of the SKC disclosed in Pupo et al. Pupo et al. merely disclose how to remove the **soluble** SKC from the insoluble cell fragments.

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Therefore, the rejection of Claims 1-5, 8-9, and 11 Under 35 USC §102(b) is untenable. Withdrawal of the same is respectfully requested.

CONCLUSION

In light of the above amendments and remarks, Applicants submit that the application is now in condition for allowance. Early notification of such action is earnestly solicited.

Respectfully submitted,

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Purification of His-tagged proteins from inclusion bodies using QuickPick™ IMAC

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KEY WORDS: His-tag, protein purification, inclusion bodies, Ni^{2+} affinity, magnetic particle separation, PickPen™, QuickPick™ IMAC

ABSTRACT

Over-expression of recombinant proteins frequently leads to the production of inclusion bodies, which are insoluble aggregates of unfolded protein. However, inclusion bodies can easily be solubilized and purified under strongly denaturing conditions.

Standard protein purification/separation methods rely on conventional liquid chromatography strategies based on chromatographic or spin columns. However, these methods are time-consuming and complex.

Bio-Nobile's QuickPick™ IMAC (Immobilized Metal Affinity Chromatography) kit can be used to carry out the purification of His-tagged proteins quickly and easily. The QuickPick methods are especially convenient for small sample volumes with emphasis on fast and material-conserving analysis.

INTRODUCTION

Genetically engineered proteins containing a histidine tail (His-tag) are widely used in both protein expression and protein function studies. In many cases and in several host systems, these recombinant proteins accumulate in cells as insoluble aggregates, so-called inclusion bodies. The proteins expressed as inclusion bodies are mostly inactive and denatured. The formation of inclusion bodies is a frequent consequence of high-level protein production in the cytoplasm. It is not possible to generalize or predict which proteins are produced as inclusion bodies.

Production of recombinant proteins as inclusion bodies has several advantages:

- The recombinant protein deposited as inclusion bodies can be 50% or more of the total cellular protein.
- Inclusion bodies often contain almost exclusively the over-expressed protein.
- In the form of inclusion bodies the protein is protected from proteolytic degradation resulting in higher yield.
- Expression as inclusion bodies will protect the cell against the toxicity of the recombinant protein since inclusion bodies have no biological activity.
- Inclusion bodies can be accumulated in the cytoplasm to a much higher level than when produced in soluble form.

The PickPen™ technology using IMAC magnetic particles has several benefits in comparison to other methods, some of the most significant being speed and ease of use. The time needed for a single purification is only 5 minutes.

The method is optimized for small sample volumes. However, with samples containing a low protein concentration volumes up to 1 ml can be used. In fact, PickPen technology also serves as a method to concentrate the target protein and thereby facilitate, for example, the detection of proteins expressed in low levels.

MATERIALS AND METHODS

QuickPick™ IMAC kit was modified to obtain denaturing conditions as follows:

IMAC Wash Buffer 1 was prepared by dissolving 1,6 g urea in 1700 µl of IMAC ^{2x}Wash Stock Buffer and adding H_2O to a final volume of 3400 µl. Final urea concentration is 8 M.

IMAC Wash Buffer 2 was prepared by dissolving 1,2 g urea in 1700 µl of IMAC ^{2x}Wash Stock Buffer, adding 135 µl of IMAC Imidazole Buffer, 500 mM, mixing well and adding H_2O to a final volume of 3400 µl. Final urea and imidazole concentrations are 6 M and 20 mM, respectively.

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EXHIBIT A

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IMAC Elution Buffer was prepared by dissolving 290 mg urea in 800 µl of IMAC Elution Buffer. Final urea and imidazole concentrations are 6 M and 240 mM, respectively.

Inclusion bodies were produced by expressing the His-tagged Btk SH_2(H362Q) protein variant in *E. coli* BL21(DE3) strain. 1.5 ml of the *E. coli* culture was centrifuged and the cell pellet was suspended in 300 µl of IMAC Wash Buffer 1 (containing 8 M urea). The suspension was sonicated for 1 min (5 s pulses and 9,9 s intervals) and after sonication the suspension was incubated for 5 minutes at room temperature with mild shaking (15 rpm) to solubilize the inclusion bodies. The suspension was centrifuged for 5 min 20 000 x g at room temperature and the clear supernatant was used as a sample.

For certain proteins the 5 minute solubilization time might be too short. However, with the Btk SH_2(H362Q) protein variant the results were almost as good using 5 minutes solubilization as with 8 hour solubilization. Also, for some proteins 6 M guanidium hydrochloride might be needed instead of 8 M urea, but guanidium hydrochloride should be used only in IMAC Wash Buffer 1 and in both cases urea should be used in IMAC Wash Buffer 2 and IMAC Elution Buffer.

As an example of the purification of recombinant protein from inclusion bodies, the procedure using the QuickPick IMAC kit is described (Fig. 1a-f). The IMAC magnetic particles were first suspended in the IMAC Regeneration Buffer (a) and then transferred into IMAC Wash Buffer 1 (containing 8 M urea) (b). The equilibrated particles were incubated for 2 min with the sample (c), washed once in IMAC Wash Buffer 2 (containing 6 M urea) (d) and to conclude the bound proteins were eluted out by incubating the particles in 25 µl of the IMAC Elution Buffer (containing 6 M urea) (e) for 1 min.

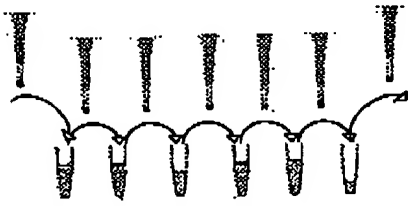

Fig. 1

The QuickPick IMAC kit reagents from Bio-Nobile were used in the purification.

RESULTS

To demonstrate the purification properties of IMAC magnetic particles with PickPen technology, the solubilized inclusion body supernatant and the final elution solution were analyzed in SDS-PAGE (Fig. 2). The gel picture shows the binding and elution behavior of proteins during the procedure. The yield of the Btk SH_2(H362Q) protein variant has been shown to be approximately 20 µg per preparation using the QuickPick IMAC kit.

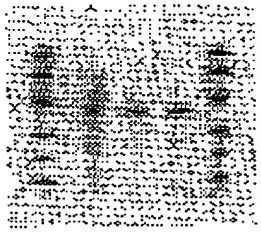

Fig. 2 SDS-PAGE gel picture

Lane 1: LMW-marker
Lane 2: 10 µl of solubilized supernatant sample, (before magnetic particle treatment)
Lane 3: 10 µl of elution buffer (5 min solubilization)
Lane 4: 10 µl of elution buffer (8 hour solubilization)
Lane 5: LMW marker

CONCLUSIONS

Proteins expressed as inclusion bodies require solubilization before purification. Urea is added to the QuickPick IMAC kit buffers to provide a simple protocol for solubilizing proteins before carrying out the purification. The QuickPick IMAC method together with PickPen transfer technology gives a fast and cost-effective means of purifying His-tagged proteins whether they are present in inclusion bodies or not.

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